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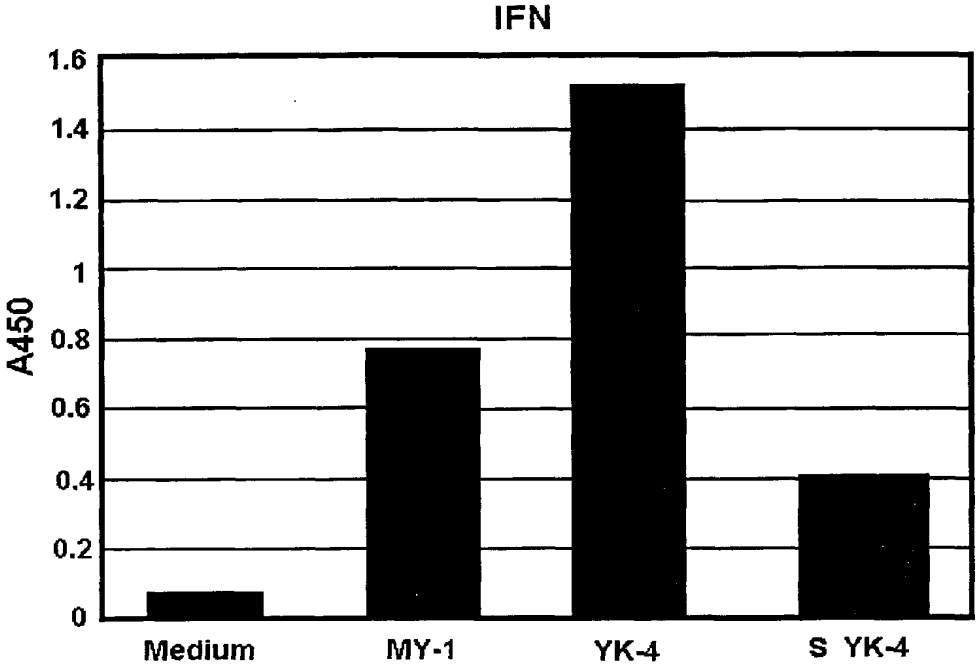

(57) Abstract: The present invention provides an oligonucleotide for stimulating an immune response in a mammal. The oligonucleotides of the invention are palindromic and flanked by up to 11 guanylic acid residues. The immune response is stimulated by administering the oligonucleotides of the invention and inducing the secretion of cytokines.

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DNA PALINDROME – OLIGOGUANYLIC ACID COMPOSITIONS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority, in part, under 35 U.S.C. §119 based upon U.S. Provisional Patent Application No. 60/215,475 filed June 30, 2000.

FIELD OF THE INVENTION

The present invention relates to the fields of molecular biology and immunology and to a DNA palindrome–oligoguanylic acid and, more particularly, to the use of DNA palindrome–oligoguanylic acid oligonucleotides for the stimulation of cytokine secretion.

BACKGROUND OF THE INVENTION

Several polynucleotides have been extensively evaluated as biological response modifiers. Perhaps the best example is poly (I,C) which is a potent inducer of IFN production as well as a macrophage activator and inducer of NK activity (Talmadge, J. E., et al., 1985 *Cancer Res.* 45:1058; Wiltrout, R. H., et al., 1985. J. *Biol. Resp. Mod.* 4:512; Krown, S. E. 1986, *Sem. Oncol.* 13:207; and Ewel, C. H., S. J., et al., 1992. *Cancer Res.* 52:3005). It appears that this murine natural killer cell (NK) activation may be due solely to induction of interferon (IFN)-β secretion (Ishikawa, R., and C. A. Biron. 1993. *J.Immunol.* 150:3713). This activation was specific for the ribose sugar since deoxyribose was ineffective. Its potent *in vitro* antitumor activity led to several clinical trials using poly (I,C) complexed with poly-L-lysine and carboxymethylcellulose (to

reduce degradation by RNAse) (Talmadge, J. E., et al., 1985. cited *supra*; Wiltrout, R. H., et al., 1985. cited *supra*); Krown, S. E., 1986. cited *supra*); and Ewel, C. H., et al., 1992. cited *supra*). Unfortunately, toxic side effects have thus far prevented poly (I,C) from becoming a useful therapeutic agent.

Guanine ribonucleotides substituted at the C8 position with either a bromine or a thiol group are B cell mitogens and may replace B cell differentiation factors (Feldbush, T. L., and Z. K. Ballas. 1985. *J. Immunol.* 134:3204; and Goodman, M. G. 1986. *J. Immunol.* 136:3335). Eight-mercaptoguanosine and 8-bromoguanosine also can substitute for the cytokine requirement for the generation of major histocompatibility complex (MHC) restricted cytotoxic T-lymphocytes (CTL) (Feldbush, T. L., 1985. cited *supra*), augment murine natural killer cell (NK) activity (Koo, G. C., et. Al., 1988. *J. Immunol.* 140:3249), and synergize with IL-2 in inducing murine lymphocyte-activated killer cell (LAK) generation (Thompson, R. A., and Z. K. Ballas. 1990. *J.Immunol.* 145:3524). The NK and LAK augmenting activities of these C8-substituted guanosines appear to be due to their induction of IFN (Thompson, R. A., et al. 1990. cited *supra*). Recently, a 5' triphosphorylated thymidine produced by a mycobacterium was found to be mitogenic for a subset of human $\gamma\delta$ T cells (Constant, P., et al., 1994. *Science* 264:267). This report indicated the possibility that the immune system may have evolved ways to preferentially respond to microbial nucleic acids.

Several observations suggest that certain DNA structures may also have the potential to activate lymphocytes. For example, Bell et al. reported that nucleosomal protein-DNA complexes (but not naked DNA) in spleen cell supernatants caused B cell proliferation and immunoglobulin secretion (Bell, D., et al., 1990. *J. Clin. Invest.* 85:1487). In other cases, naked DNA has been reported to have immune effects. Aside from such artificial homopolymer sequences (*supra*), it has been reported that pure mammalian DNA has no detectable immune effects, but that DNA from certain bacteria induces B cell activation and immunoglobulin secretion (Messina, J. P., et al., 1991. *J.*

Immunol. 147:1759). Assuming that these data did not result from some unusual contaminant, these studies suggest that a particular structure or other characteristic of bacterial DNA renders it capable of triggering B cell activation. Investigations of mycobacterial DNA sequences have demonstrated that oligonucleotides that contain certain palindrome sequences can activate NK cells (Yamamoto, S., et al., 1992. *J. Immunol.* 148:4072; Kuramoto, E., O. et. al., 1992. *Jpn. J. Cancer Res.* 83:1128). Other investigators have shown that a palindrome structure within single stranded DNA is responsible for the induction of cytokines. (Sonehara, K., et al., *J. Interferon and Cytokine Res.* 16:799-803, 1996). The induction of IFN α secretion by single stranded phage M13 DNA (*Antivir. Res.* 31:79-86) has been shown to result in antiviral activity, as revealed by the ability of phage M13 DNA to induce a strong anti-Hepatitis B virus response. (Iizuka, A., et al.,*Hepatology* 19:1079-1087, 1994).

Several phosphorothioate modified oligonucleotides have been reported to induce *in vitro* or *in vivo* B cell stimulation (Tanaka, T., et al., 1992. J. *Exp. Med.* 175:597; Branda, R. F. , et al., 1993. *Biochem. Pharmacol.* 45:2037; McIntyre, K. W., et al., 1993. *Antisense Res.* Develop. 3:309; and Pisetsky, D. S., and C. F. Reich. 1993. *Life Sciences* 54:101). These reports do not suggest a common structural motif or sequence element in these oligonucleotides that might explain their effects.

Another oligonucleotide sequence that has been shown to induce an immune response is deoxyguanylic acids. Deoxyguanylic acids, but not other deoxynucleotides as short as 3 to 4 nucleotides, were effective in preventing HIV-1-induced cytopathicity. (Fujihashi, T., et. al., 1995a, *Antimicrob Agents Chemother.* 39:2000-2007; Fujihashi, T., et. al., 1994, *Biochem. & Biophys. Res. Commun.* 203:1244-1250; Fujihashi, T., et. al.,1995b, *AIDS Res. and Hum. Retroviruses* 11:461-471).

The present invention provides a more efficacious oligonucleotide for induction of cytokine secretion wherein a palindrome is flanked on at least one side by deoxyguanlyic acid. The present invention relates to methods and

compositions for stimulating an immune response in a mammal. The present invention further provides pharmaceutcal compositions containing the oligonucleotide of the present invention.

ABBREVIATIONS

"IFN" means "interferon"

"NK" means "natural killer T-lymphocytes"

"SPC" means "spleen cells"

"BMC" means "mouse bone marrow cells"

"PBMC" means "peripheral blood monocytes"

"G4-PAL-G4" means "a palindrome flanked by tetraguanylic acid"

"G4-AACGTT-G4" means "a palindrome flanked by G4 on both 5' and 3' sides, the internucleotide linkage being a phosphodiester linkage"

"pG4-AACGTT-G4" menas "5' phosphorylated G4-AACTGTT-G4'

"PS G4-AACGTT-G4" means "phosphorothio G4-AACGTT-G4, the internucleotide linkage being a phosphothiate linkage"

"CPE" means "cytopathic effect"

"PEC" means "periotneal exudate cells"

DEFINITIONS

"G4-palindrome combination" is meant to be synonymous to G4-PAL-G4, which is a palindrome flanked by tetraguanylic acid.

"guanylic acid-palindrome" is meant to mean a palindrome flanked on one or both sides by no more than 11 guanylic acid residues.

"YK-4" is meant to mean G4-AACGTT-G4

"S-YK-4" is meant to mean phosphorothioate G4-AACGTT-G4

"MY-1" is meant to mean single stranded DNA prepared from *M. tuberculosis*

"palindrome" is meant to mean an inverted repeat (i.e. a sequence such as

ABCDEE'D'C'B'A' in which A and A' are bases capable of forming the usual Watson-Crick base pairs. *In vivo,* such sequences may form double-stranded structures.

"flanking" is meant to mean sequences adjacent to the palindrome sequence on the 5' and/or 3' sides.

DESCRIPTION OF THE DRAWINGS

Figure 1. IFN activity is assessed by the inhibition of the cytopathic effect (CPE) of vesicular stomatitis virus on mouse L929 fibroblast cells. The antiviral activity of the reference IFN or the fluid samples is detected as CPE inhibition of the viable cell counting method, as determined by tetrazolium (WST-1 and 1-methoxy PMS; Dojindo, Japan) uptake. Briefly, the L929 fibroblast cells are incubated with the virus for 2 hours and the optical density (OD) is measured with a microplate reader at 450 nm. The absorption value of the cells stimulated with 100 U/ml of the reference IFN is 1.3.

Figure 2. Stimulation of NK cell activity by the G4-palindrome-G4 oligonucleotide. BALB/c mice (5 mice / experimental group) weighing 20-25 grams are injected intraperitoneally (i.p.) with 100 μg of MY-1, YK-4 or S-YK-4. Twenty-four hours later the peritoneal exudate cells (PEC), containing the NK cells, are collected from the mice. ^{51}Cr-labeled RL male1 (a cell line from BALB/c mice) target cells are mixed with PEC, four hours later the NK cell activity is assessed as a percent lysis of the ^{51}Cr-labeled target cells.

DESCRIPTION OF THE INVENTION

Interferon (IFN) and other cytokines are very important in the treatment of various diseases, such as cancer and viral infection. Presently most of the

cytokine treatment is performed by external administration of the appropriate cytokines. The invention disclosed herein presents an alternative to the external administration of cytokines by providing oligonucleotides flanked by oligoguanylic acids for the efficacious induction of cytokines.

The present invention examines the enhancing effects of palindrome-tetraguanylic acid oligonucleotides on induction of cytokines. The present invention shows that the addition of tetraguanylic acid (tetra G) on either end of the palindrome structure (G4PALG4) results in a 1000-fold stimulation of cytokine induction by the palindrome.

Therapeutic/prophylactic uses

Phagocytosis is a major host defense mechanism by which potentially deleterious material (both pathogenic organisms and cellular debris) is cleared from circulation and tissue, and made accessible to inactivation. The phagocytic capacity/potential of a cell is modulated by cytokines, which trigger cellular differentiation.

The mechanism for the antiviral and antitumor activity of immunostimulating compounds is thought to be due in substantial part to enhancement of the immune response due to induction of various important cytokines (e.g., interferons, interleukins, tumor necrosis factor, etc.). Such compounds have been shown to stimulate a rapid release of certain monocyte/macrophage-derived cytokines and to be capable of stimulating B cells to secrete antibodies which play an important role in an immunostimulating compounds' antiviral and antitumor activities. One of the predominant immunostimulating responses to immunostimulating compounds is the induction of interferon IFN α production, which is believed to be very important in acute antiviral and antitumor activities. Moreover, up regulation of other cytokines such as, for example, tumor necrosis factor (TNF), IL-1 and IL-6 also have a potentially beneficial effect, as cytokines are believed to contribute to the antiviral and antitumor properties of immunostimulating compounds.

In stimulating an immune response by the oligonucleotides of the present invention, the subject is first exposed to the antigen. As used herein, the term "exposed to" refers to either the step of contacting the subject with an antigen or the exposure of the subject to the antigen as would occur naturally *in vivo.* Methods for the administration of an antigen to a subject are well-known in the art. In general, an antigen is administered directly to the subject by any means such as, but not limited to, intravenous, intramuscular, oral, transdermal, mucosal, intranasal, intratracheal, or subcutaneous administration. The antigen can be administered systemically or locally. A subject is exposed to an antigen if an antigen becomes available for exposure to the immune cells in the body. A subject may be exposed to an antigen, for instance, by entry of a foreign pathogen into the body or by the development of a tumor cell expressing a foreign antigen on its surface.

The timing of guanylic acid-palindrome combination oligonucleotide administration can be dependent on a subject's anticipated exposure to an antigen. For instance, in a subject at risk of developing a cancer or an infectious disease or an allergic or asthmatic response, the subject may be administered the guanylic acid-palindrome combination oligonucleotide on a regular basis when that risk is greatest, i.e., during allergy season or after exposure to a cancer causing agent. Additionally the guanylic acid-palindrome combination oligonucleotide may be administered to travelers before they travel to foreign lands where they are at risk of exposure to infectious agents.

The invention is useful for treating a mammal that is capable of developing cancer, infections, allergies, and asthma. A mammal includes, but is not limited to, bovines, ovines, porcines, equines, rodents and humans. In addition to the treatment of infectious human diseases, the methods of the invention are useful for treating infections of animals.

As used herein, the term "treat", "treated", or "treating" when used with respect to an infectious disease refers to a prophylactic treatment that increases the resistance of a subject to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen

as well as a treatment after the subject has become infected in order to fight the infection, e.g., reduce or eliminate the infection or prevent it from becoming worse.

Thus, the present invention contemplates the use of guanylic acid-palindrome oligonucleotides to induce an antigen specific immune response in a mammal. Antigens include, but are not limited to, infectious microbes such as viruses, bacteria and fungi and fragments thereof, derived from natural sources or synthetically. Infectious viruses of both human and non-human vertebrates, include but are not limited to, retroviruses, RNA viruses and DNA viruses. The oligonucleotides of the present invention are also useful for treating allergic diseases, wherein the allergen is an antigen including, but not limited to, pollens, dust, fungal spores, drugs (such as penicillin), etc. A mammal at risk for developing a cancer, or one who has cancer, can also be treated according to the methods of the present invention. The list of antigens related to infectious diseases, cancer or allergies is enormous, and it is beyond the scope of the present invention to list them, as they are well known to those of skill in the art. The stimulation of an immune response with the guanylic acid-palindrome oligonucleotides of the present invention is contemplated to be useful for the treatment of any disease or disorder associated with an antigen.

The amount of guanylic acid-palindrome oligonucleotide that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition and is determined by standard clinical techniques. The "effective amount" of the guanylic acid-palindrome oligonucleotide is that which is necessary or sufficient to stimulate an immune response. The precise dose to be employed in the formulation is also dependent on the route of administration, the seriousness of the disease or disorder and is decided according to the judgement of the practitioner and each patient's circumstances. For use in therapy, an effective amount of a guanylic acid-palindrome oligonucleotide alone or formulated as a nucleic acid delivery complex can be administered to a subject by any mode allowing the oligonucleotide to be taken up by the appropriate target cells (for instance, but

not limited to, monocytes/macrophages, spleen cells, etc). Routes of administration include, but are not limited, to oral, transdermal (e.g. via a patch), injection (subcutaneous, intravenous, parenteral, intraperitoneal, intrathecal, etc.), intranasal, intratracheal, and mucosal. An injection may be in a bolus or a continuous infusion. Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the compositions of the invention. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

While this invention is described with a reference to specific embodiments, it is obvious to those of ordinary skill in the art that variations in these methods and compositions (such as the type of antigen against which an immune response is to be directed against and the length of guanylic acids flanking the palindrome) may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims.

Pharmaceutical compositions

Pharmaceutical compositions may contain suitable buffering agents, including, but not limited to: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the compositions of the invention, which is isotonic with the blood of the recipient. This aqueous preparation is formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation is a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent,

for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including, but not limited to, synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular composition selected, the severity of the condition being treated and the dosage required for therapeutic efficacy (*supra*). The methods of the invention are practiced using any mode of administration that is medically acceptable (*supra*), meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the compositions of the invention into association with a carrier which constitutes one or more accessory ingredients (*supra*). In general, the compositions are prepared by uniformly and intimately bringing the compositions of the invention into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Oligonucleotide modifications

For use *in vivo*, nucleic acids are formulated to be relatively resistant to degradation (e.g. via endo- and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications (*infra*). A stabilized nucleic acid can have at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized

using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl- and alkyl-phosphonates can be made for example as described in U.S. Pat. No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A., 1990, Chem. Rev. 90:544; Goodchild, J., 1990, Bioconjugate Chem. 1:165). 2'-O-methyl nucleic acids will also cause immune activation, as do ethoxy-modified nucleic acids.

Modified oligonucleotides include, but are not limited to, phosphodiester modified oligonucleotide, combinations of phosphodiester and phosphorothioate oligonucleotide, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof. It is believed that these modified oligonucleotides may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization. Other stabilized oligonucleotides include, but are not limited to: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation. The aforementioned list of modifications is not meant to be limiting, for it is well known to those skilled in the art what modifications, if any, are to be included in the guanylic acid-palindrome oligonucleotides of the present invention.

Materials and Methods

Interferon (IFN) α/β, γ are measured as described previously (*Antivir. Res.* 31:79-86). Mouse peripheral blood monocytes (PBMC), mouse bone

marrow cells (BMC) or mouse spleen cells (SPC) are suspended to a concentration of 10^7/ml of tissue culture media. They are exposed to the oligonucleotides as described in each experiment for 24 hrs at 37° C. The IFN is measured by the capacity of the tissue culture fluid to prevent the cytopathic effect of vesicular stomatitis virus (VSV) on mouse L-929 cells. An ELISA assay is used to measure TNF, IL-6 and IL-12.

To measure cell lysis by the NK cells, RL male1 (a cell line from BALB/c mice) target cells (Shimada, S., et al., *Jpn. J. Cancer Res.* 77:808-816, 1986) are first loaded with ^{51}Cr, this labeling of the target cells with ^{51}Cr is well known to those of skill in the art, and can be found in standard manuals (Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.) as well as in Yamamoto, et al. (Yamamoto, S., et. al., *J. Immunol.* 148: 4072, 1992).

Results

The capacity of the palindrome to induce cytokine secretion is enhanced by flanking the palindrome sequence on at least one side with tetra G. In **Table 1**, production of IFN α/β by BMC and SPC by deoxynucleotide (lines 1-5), by 5' phosphorylated deoxynucleotide (lines 6-10), and by phosphorothioate derivatives (lines 11-15) are shown. It is clear that the palindrome flanked by G4 on both sides results in a greater stimulation of α/β IFN by BMC and SPC.

MY-1 is the single stranded DNA prepared from *M. tuberculosis* that has been shown to have anti-tumor activity (Tokunaga, T., et al., *J. Natl. Cancer. Instit.* 72: 955-962, 1984 and Shimada, S., et al., *J. Natl. Cancer. Instit.*74: 681-688, 1985).

The palindrome flanked by G4 on the 5' and 3' sides results in a 16-fold increase in secretion of IFN α/β by SPC relative to that secreted by induction with MY-1. The IFN α/β induction in SPC by the oligonucleotides of the present invention, on a weight-by-weight comparison, is 30 times greater than that of MY-1.

The *in vivo* efficacy of the palindrome flanked by G4 on both sides (YK-4) is shown in **Figure 1**. The YK-4 oligonucleotide has a 2-fold greater IFN (including α, β, and γ) stimulatory activity than MY-1 and a 4-fold greater stimulatory activity than the phosphorothioate palindrome flanked by G4 on both sides (S-YK-4). Thus, the enhancing effects on cytokine stimulation *in vivo* by oligonucleotides of the present invention will augment the immune response against an antigen.

The efficacy of oligonucleotides of the present invention is further supported by the stimulatory activity of NK cells by the oligonucleotides of the present invention. NK cells are natural killer cells that are stimulated to kill a specific target cell (the example used herein, which is not meant to limit the invention, is the RL male1 cell line derived from BALB/c mice), resulting in cell lysis. This is very important in treating and/or inhibiting cancers, as well as viral, parasitic and any other infection that requires a cellular immune response. **Figure 2** shows that YK-4 has 2-3 fold greater activity in stimulating NK activity than either MY-1 or the phosphorothiate version of the G4-palindrome-G4 (S-YK-4). The ability of the oligonucleotides of the present invention to stimulate both cytokine secretion and NK activity *in vivo* allows for the enhancement of a specific immune response to an antigen.

Table 2 shows a similar effect on induction of IFN γ by the G4-palindrome combination. Upon closer examination of the oligonucleotides of the present invention, the phosphodiester G4-palindromes, it is clear that on a weight-by-weight basis the phosphodiester G4-palindrome stimulates IFN γ induction 420 times greater than that of MY-1. In both SPC and BMC, the IFN induction is greater with phosphorothioate G4-PAL-G4 than MY-1. In SPC the phosphorothioate G4-PAL-G4 is just as effective as the phosphodiester counterpart. The efficacy with which IFN γ is induced with the G4-palindrome combination reveals that the oligonucleotides of the present invention will substitute for administration of isolated cytokines.

IL-6 is a multifunctional immunoregulatory cytokine acting on T cells and spleen cells. It also affects the differentiation of neuronal and B cells. It is also known that IL-6 is an essential co-factor for IgE production, the blood concentration of which depends on the patient's immunological status. **Table 3** shows that the induction of IL-6 by G4-PAL-G4 is 6-10 times higher than that of MY-1.

IL-12 is known to induce cytokines, particularly IFN γ, in T cells and NK cells. It is also known that bacterial cell wall LPS will stimulate the induction of IL-12, which subsequently stimulates IFN γ secretion, in monocytes/macrophages. **Table 4** shows IL-12 is only induced with the palindrome flanked by G4 on both ends. It should be noted that stimulation of IL-12 in BMC and SPC by phosphorothioate G4 palindrome does not induce secretion of IL-12. As other cytokines are induced by phosphorothioate G4-PAL-G4 this result is not attributed to toxicity of the oligonucleotide.

Table 5 shows the induction of TNF α by the G4-palindrome combination. The G4-palindrome combination is at least l0-fold more effective than MY-1, implying the greater efficacy of the G4-palindrome combination over naturally occurring single stranded DNA. TNF α is one of the cytokines important for defense against bacterial and viral infections. Thus, the administration of the oligonucleotides of the present invention allows for the appropriate and endogenous production of TNF α, thereby resulting in the treatment of various diseases.

Table 1: Tetra G on the 5' and 3' ends of a palindrome increases the palindromes capacity to induce IFN α/ß production by BMC and SPC.

Line #	Oligo Sequence	Concentration		IFN α/β(μg/ml)	
		μM	μg/ml	BMC	SPC
1	G4-AACGTT-G4	4	18.6	12800	3200
2	G4-AACGTT	4	13	800	400
3	AACGTT	4	7.5	6.25	6.25
4	G8	4	10.8	6.25	6.25
5	G4	4	5.2	6.25	6.25
6	p G4-AACGTT-G4	4	18.9	12800	3200
7	p G4-AACGTT	4	13.4	6.25	6.25
8	p AACGTT	4	7.8	6.25	6.25
9	p G8	4	11.1	6.25	6.25
10	p G4	4	5.5	6.25	6.25
11	PS G4-AACGTT-G4	4	18.5	3200	50
12	PS G4-AACGTT	4	13	6400	100
13	PS AACGTT	4	7.5	800	6.25
14	PS G8	4	10.7	6.25	6.25
15	PS G4	4	5.2	6.25	6.25
16	MY-1		40	3200	200
17	Medium			6.25	6.25

Table 2: Tetra G on the 5' and 3' ends of a palindrome increases the palindromes capacity to induce IFN γ production by BMC and SPC.

Line #	Oligo Sequence	Concentration		IFNγ (μg/ml)	
		μM	μg/ml	BMC	SPC
1	G4-AACGTT-G4	4	18.6	10200	19400
2	G4-AACGTT	4	13	369	6070
3	AACGTT	4	7.5	1	1
4	G8	4	10.8	71	1
5	G4	4	5.2	173	1
6	p G4-AACGTT-G4	4	18.9	11500	21230
7	p G4-AACGTT	4	13.4	1	1
8	p AACGTT	4	7.8	1	1
9	p G8	4	11.1	1	7.5
10	p G4	4	5.5	1	38.5
11	PS G4-AACGTT-G4	4	18.5	2280	17400
12	PS G4-AACGTT	4	13	2050	14600
13	PS AACGTT	4	7.5	1	1
14	PS G8	4	10.7	1	146
15	PS G4	4	5.2	129	208
16	MY-1		40	1150	9000
17	Medium			42.1	71

Table 3: Tetra G on the 5' and 3' ends of a palindrome increases the palindromes capacity to induce IL-6 production by BMC and SPC.

Line #	Oligo Sequence	Concentration		IL-6 μg/ml)	
		μM	μg/ml	BMC	SPC
1	G4-AACGTT-G4	4	18.6	4410	2540
2	G4-AACGTT	4	13	552	357
3	AACGTT	4	7.5	185.5	5.9
4	G8	4	10.8	53.3	96.4
5	G4	4	5.2	96.4	117
6	p G4-AACGTT-G4	4	18.9	6810	2870
7	p G4-AACGTT	4	13.4	11	141
8	p AACGTT	4	7.8	67.9	2.9
9	p G8	4	11.1	31.3	1
10	p G4	4	5.5	218	1
11	PS G4-AACGTT-G4	4	18.5	2230	1440
12	PS G4-AACGTT	4	13	1500	805
13	PS AACGTT	4	7.5	178	1
	PS G8	4	10.7	11	44
	PS G4	4	5.2	92	113
16	MY-1		40	1280	853
17	Medium			15.1	125

Table 4: Tetra G on the 5' and 3' ends of a palindrome increases the palindromes capacity to induce IL-12 production by BMC and SPC.

Line #	Oligo Sequence	Concentration		IL-12 (μg/ml)	
		μM	μg/ml	BMC	SPC
1	G4-AACGTT-G4	4	18.6	69	64.88
2	G4-AACGTT	4	13	1	1
3	AACGTT	4	7.5	1	1
4	G8	4	10.8	1	1
5	G4	4	5.2	1	1
6	p G4-AACGTT-G4	4	18.9	196	88.58
7	p G4-AACGTT	4	13.4	1	1
8	p AACGTT	4	7.8	1	1
9	p G8	4	11.1	1	1
10	p G4	4	5.5	1	1
11	PS G4-AACGTT-G4	4	18.5	1	1
12	PS G4-AACGTT	4	13	6.1	1
13	PS AACGTT	4	7.5	1	1
14	PS G8	4	10.7	1	1
15	PS G4	4	5.2	1	1
16	MY-1		40	1	1
17	Medium			1	1

Table 5: Tetra G on the 5' and 3' ends of a palindrome increases the palindromes capacity to induce TNFα production by BMC and SPC.

Line #	Oligo Sequence	Concentration		TNFα (μg/ml)	
		μM	μg/ml	BMC	SPC
1	G4-AACGTT-G4	4	18.6	2910	2600
2	G4-AACGTT	4	13	159	134
3	AACGTT	4	7.5	47.6	61.9
4	G8	4	10.8	51.9	74.3
5	G4	4	5.2	27.7	51.3
6	p G4-AACGTT-G4	4	18.9	4830	3210
7	p G4-AACGTT	4	13.4	28.5	84.8
8	p AACGTT	4	7.8	30.8	83
9	p G8	4	11.1	19.7	73.7
10	p G4	4	5.5	24	57.5
11	PS G4-AACGTT-G4	4	18.5	2360	546
12	PS G4-AACGTT	4	13	1080	373
13	PS AACGTT	4	7.5	107	69.3
14	PS G8	4	10.7	33.3	116
15	PS G4	4	5.2	32.7	135
16	MY-1		40	457	243
17	Medium			28.4	69.3

CLAIMS

What is claimed is:

1. A guanylic acid-palindrome comprising a palindrome flanked on at least one side by no more than 11 guanylic acid residues.

2. A guanylic acid-palindrome comprising a palindrome flanked on at least one side by 4 guanylic acid residues.

3. An immunogenic oligonucleotide comprising, a guanylic acid-palindrome comprising a palindrome flanked on at least one side by no more than 11 guanylic acid residues.

4. An immunogenic oligonucleotide comprising, a guanylic acid-palindrome comprising a palindrome flanked on at least one side by 4 guanylic acid residues.

5. A pharmaceutical composition comprising any one of Claims 1-4.

6. A method for stimulating an immune response in a mammal comprising:
   a) administering to said mammal exposed to an antigen an effective amount of a guanylic acid-palindrome wherein said guanylic acid comprises no more than 11 guanylic acid recidues; and
   b) inducing secretion of at least one cytokine.

7. The method of Claim 6 wherein said antigen comprises at least one of a tumor antigen, a viral antigen, an allergen or a microbial antigen.

8. The method of Claim 6 wherein said cytokine comprises at least one of IFN α/β, IFN γ, IL-6, IL-12, or TNF α.

9. The method of Claim 6 wherein said guanylic acid-palindrome comprises a palindrome flanked on at least one side by 4 guanylic acid residues.

10. A method for stimulating an immune response in a mammal comprising:

a) administering to said mammal exposed to an antigen an effective amount of a palindrome flanked on at least one side by 4 guanylic acid residues; and

b) inducing secretion of at least one cytokine.

11. The method of Claim 10 wherein said antigen comprises at least one of a tumor antigen, a viral antigen, an allergen or a microbial antigen.

12. The method of Claim 10 wherein said cytokine comprises at least one of IFN α/β, IFN γ, IL-6, IL-12, or TNF α.

13. A method for stimulating an immune response in a mammal comprising:

a) administering to said mammal exposed to an antigen an effective amount of a palindrome flanked on at least one side by 4 guanylic acid residues, wherein said antigen is any one of a tumor antigen, a viral antigen, an allergen or a microbial antigen; and

b) inducing secretion of at least one of IFN α/β, IFN γ, IL-6, IL-12, or TNF α.

14. A guanylic acid-palindrome comprising a palindrome portion of AACGTT flanked on at least one side by up to 11 guanylic acid residues.

15. A guanylic acid-palindrome comprising a palindrome portion of AACGTT on at least one side by 4 guanylic acid residues.

16. A pharmaceutical composition comprising at least one of Claim 14 or Claim 15.

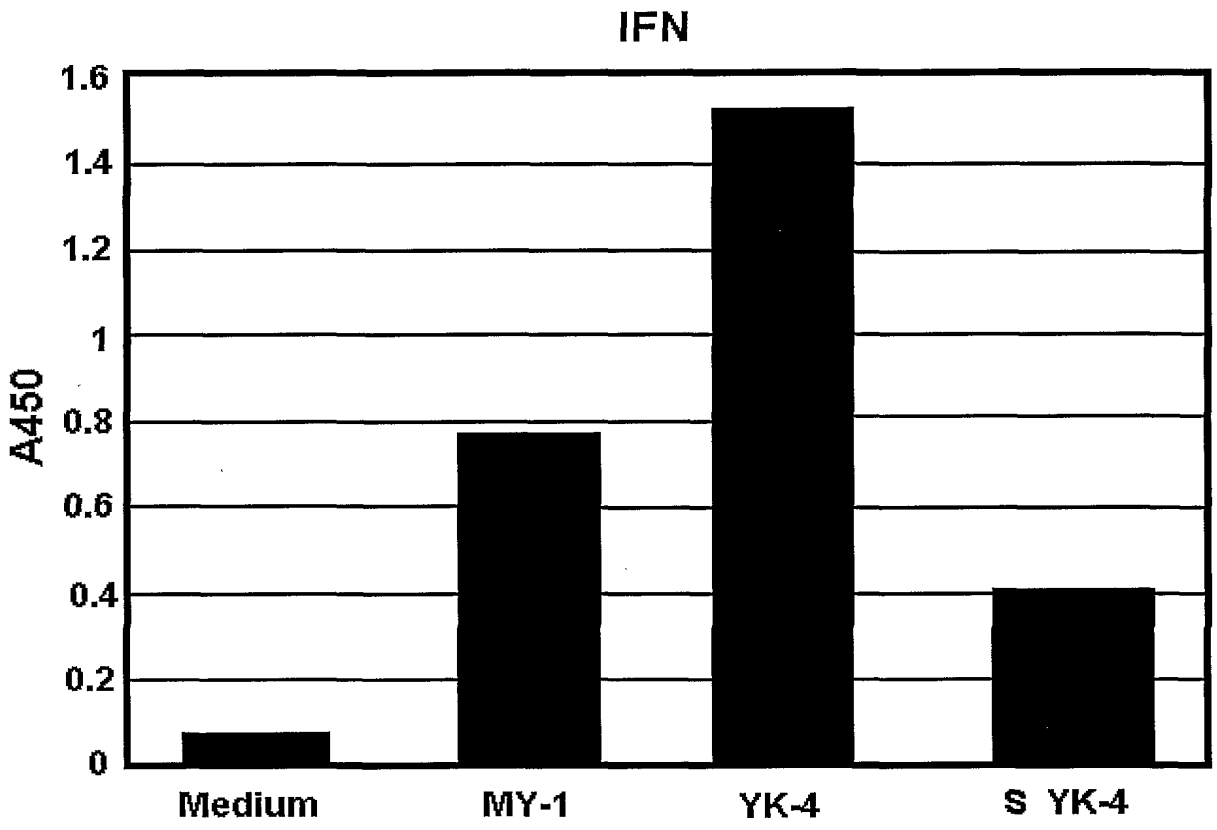

Fig. 1

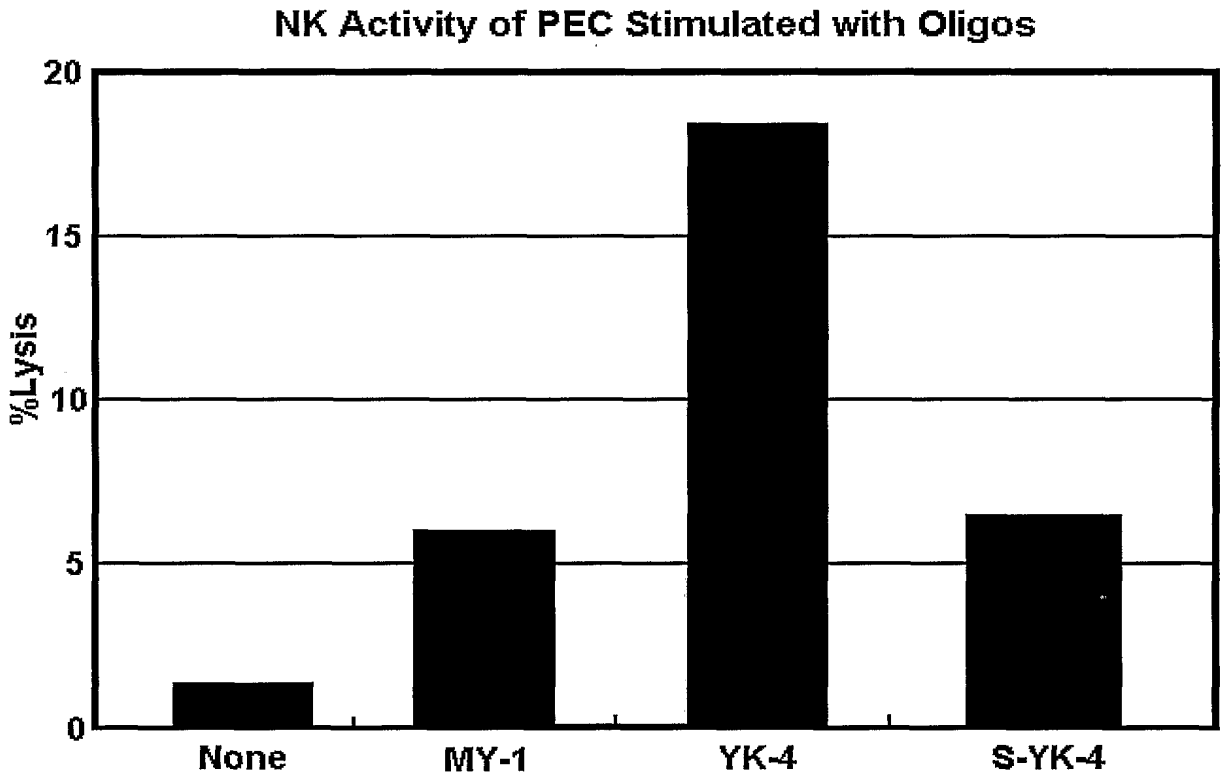
NK Activity of PEC Stimulated with Oligos
%Lysis
20
15
10
5
0
None
MY-1
YK-4
S-YK-4

Fig. 2

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 01/20426

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61M25/00 A61B17/43 A61D19/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61M A61B A61D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 840 623 A (QUACKENBUSH JOHN) 20 June 1989 (1989-06-20) column 2, line 3 -column 3, line 11; figures	1,2,9,18
X	EP 0 161 863 A (WARNER LAMBERT CO) 21 November 1985 (1985-11-21) page 10, line 16 -page 13, line 10; figures	1,9-11
A	EP 0 631 791 A (TERUMO CORP) 4 January 1995 (1995-01-04) page 5, line 41 -page 6, line 25; figures	1,3-7, 18-20
A	US 6 030 369 A (CARTER MARK ET AL) 29 February 2000 (2000-02-29) column 3, line 57 -column 5, line 40; figures	1-11

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 2001	19/11/2001

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/US 01/20426

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